Luc-Pair™ miR Luciferase Assay

For successive luciferase assays

Cat. No. LPFR-M010 (100 reactions)
Cat. No. LPFR-M030 (300 reactions)
Cat. No. LPFR-M100 (1000 reactions)

User Manual

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USER MANUAL

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I. Introduction and Principle

The study of transcriptional regulation using reporter gene expression is common in cell biology research and pharmaceutical discovery. In earlier years, research focused on the regulation of promoter activity. Recently, post-transcriptional regulations have been identified as another gene regulatory mechanism found in cells. For example, small inhibitory RNAs, such as siRNA and miRNA, have been implicated in a wide range of post-transcription cellular functions.

Luciferase is the most widely used genetic reporter for gene expression studies due to several advantages including the following:

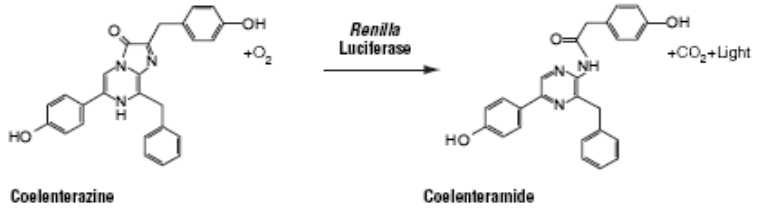
1) high sensitivity in a large dynamic range
2) natural absence from mammalian cells
3) consistent reproducibility
4) cost effectiveness
5) simple assay format
6) suitable for HTS

Firefly (*Photinus pyralis*) luciferase has been proven to be an ideal reporter for monitoring both promoter activity and post-transcriptional regulations in the control of gene expression. It is a cytoplasmic enzyme with a molecular weight at about 61 kDa and catalyzes the following reaction:

$$\text{ATP + luciferin + } O_2 \xrightarrow[\text{Mg++}]{\text{Luciferase}} \text{Oxyluciferin + AMP + PPi + } CO_2 \text{ + LIGHT}$$

The intensity of light emission is linearly related to the amount of luciferase and is measured using a luminometer and luminescence plate reader.

Renilla (Renilla reniformis) luciferase is a 36 kDa protein. It is a monomeric protein and requires no post-translational processing. It can function as a genetic reporter immediately upon translation. It catalyzes the following reaction:



In general, it is relatively simple to monitor the up regulation of genetic elements with firefly luciferase assays. It has been more difficult to measure the down regulation of genes since assay windows are relatively small for down regulation and some non-specific events can reduce the luciferase activity, such as cell death. Therefore, normalizing the expression of an experimental reporter to the expression of an independent control reporter can help differentiate between specific and nonspecific cellular responses. Normalization is also needed for adjusting differences in transfection efficiencies and cell viability.

Firefly and *Renilla* luciferases have been widely used as co-reporters for normalization studies because both assays are quick, easy and sensitive. Firefly and *Renilla* luciferases are ideal co-reporters because they have distinct evolutionary origins and very different enzyme structures and substrates.

GeneCopoeia has leveraged the differences in firefly and *Renilla* enzyme structures and substrates to optimize and develop a convenient system for measuring two luciferase activities in succession. The assay measures the activities of firefly and *Renilla* luciferases sequentially from a single sample. The firefly luciferase luminescence is elicited by one reagent, while a second reagent simultaneously quenches the firefly luciferase and elicits *Renilla* luciferase luminescence.

GeneCopoeia Luc-Pair miR Luciferase Kit development team incorporated several features and benefits into the reagents to enhance product performance and add convenience. Users should enjoy the following:

- The system has been designed for use on different mammalian cells and is optimized for use on micro-plate readers for high-throughput screening assays. Single-tube samples can also be used.
- The system produces very limited background luminescence (Figure 1). No subtraction is required from readings.
- The Working Solution I (Solution I combined with Substrate I) has all the ingredients necessary for lysing cells in addition to the substrates and stabilizers for the firefly luciferase reaction in a single solution.
- *Renilla* luciferase buffer contains the ingredients to quench firefly luciferase activity from the first step (Figure 2).
- The reagents have been developed so that the signals for firefly and *Renilla* luciferases are relatively stable (Figure 3). Thirty minutes after addition of the appropriate reagent, firefly activity will be at least 80% of its initial activity at 22°C. *Renilla* luciferase activity will be at least 80% of its initial activity after 15 minutes.
- This system is designed to yield reliable, linear results for a concentration range over several orders of magnitude (Figure 4).

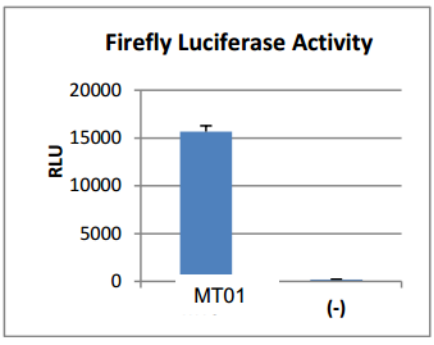


Figure 1a

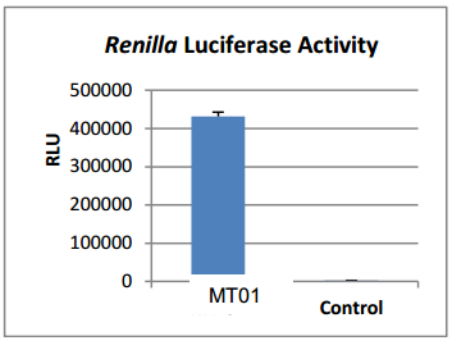


Figure 1b

Figure 1. The background of firefly luciferase is low with GeneCopoeia Luc-Pair miR Luciferase Assay. HEK293 cells were plated on a 6-well plate. On the second day, the cells were transfected with either GeneCopoeia pEZX-MT01 target reporter vector or a control plasmid. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. Then both luciferase assays were performed as described in the procedure. Firefly luciferase activity was measured first (Fig. 1a), followed by *Renilla* luciferase activity (Fig. 1b). Similar results have been obtained on CHO-K1 cells and HeLa cells (Data not shown).

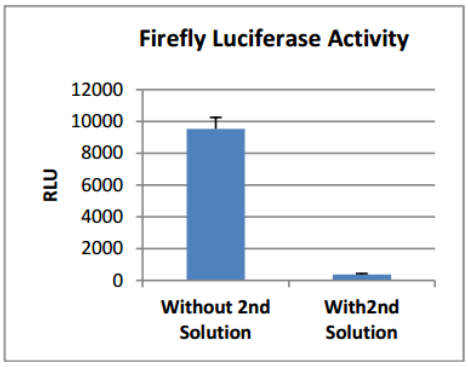


Figure 2. Firefly luciferase activity is quenched with the addition of Solution II. HEK 293 cells were plated on a 6-well plate. On the second day, the cells were transfected with GeneCopoeia pEZX-MT01 miRNA reporter vector. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. The firefly luciferase activity was measured in each well of the 96-well plate as described in the procedure. Immediately afterwards, Solution II (without Substrate II) was added into the wells of the right-half of the plate. The whole plate was measured again on a Victor II machine. About 95% of firefly luciferase activity was quenched.

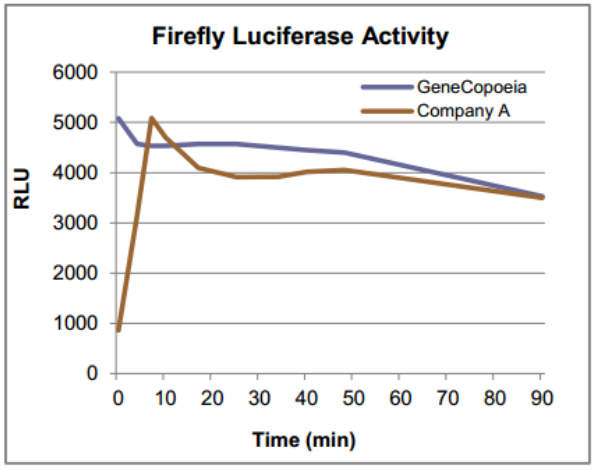


Figure 3a

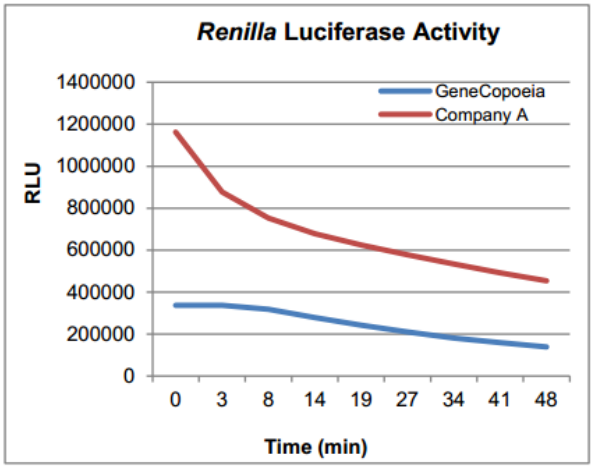


Figure 3b

Figure 3. Stability of firefly luciferase and *Renilla* luciferase signals using GeneCopoeia Luc-Pair miR Luciferase Assay. HEK293 cells were plated on a 6-well plate. On the second day, the cells were transfected with GeneCopoeia pEZX-MT01 miRNA reporter vector. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. Luciferase assays were then performed as described in the procedure and data was recorded on Victor II machine at different time points. The Company A's two-luciferase assay kit was used as control. Figure 3a: stability of the signal from firefly luciferase activity; Figure 3b: stability of the signal from *Renilla* luciferase activity.

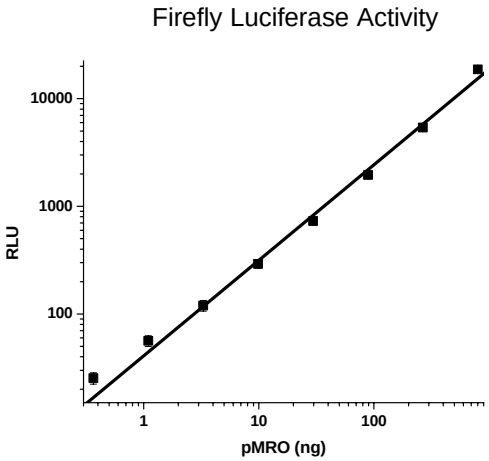


Figure 4a

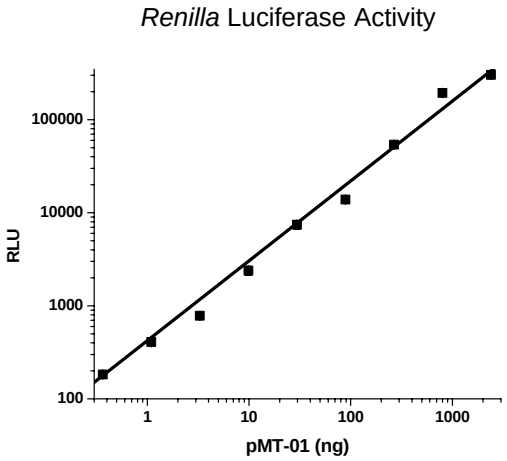


Figure 4b

Figure 4. Linear relationship between emitted light and amount of luciferase expression vector transfected in HEK293. HEK 293 cells were plated on a 6-well plate. On the second day, the cells were transfected with different amounts of GeneCopoeia pMRO or pEZX-MT01 miRNA 3' UTR Target Sequence Clone as indicated in the figure. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. Firefly luciferase assay was then performed on pMRO vector (Fig. 4a) and *Renilla* luciferase assay was performed on pEZX-MT01 vector (Fig. 4b) as described in the procedure.

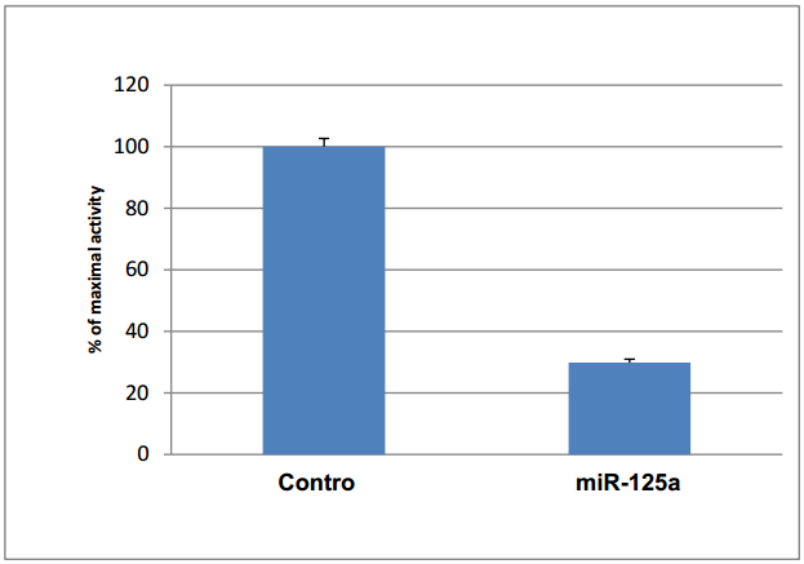


Figure 5. The inhibitory effect of miRNA on a target sequence (3' UTR) expression clone can be measured with GeneCopoeia Luc-Pair miR Luciferase Assay kit. HEK 293 cells were plated on a 6-well plate. On the second day, the cells were transfected with 1.0 μg of target sequence (3' UTR) expression clone (pEZX-MT01-Lin28 UTR-fLuc) and 1.4 μg of miRNA expression vector (or miRNA control vector) as indicated in the figure. The cells were transferred to a 96-well plate 18 hours after transfection and cultured for another 24 hours. Both firefly luciferase and *Renilla* luciferase activities were measured as described in

the procedure and data was recorded on Victor II machine. Firefly luciferase activity was then normalized with *Renilla* luciferase activities in the same well.

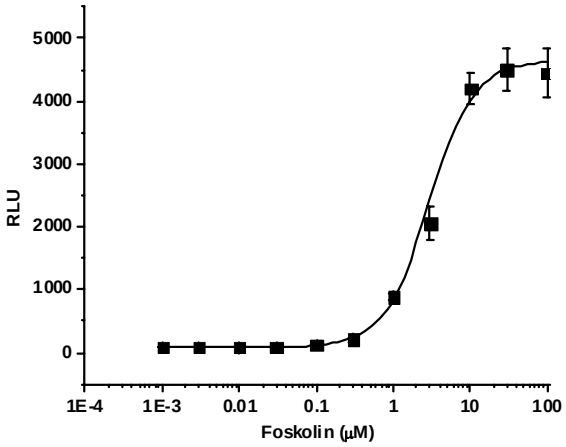


Figure 6. Up-regulation of CRE-dependent luciferase expression by foskolin in HEK293 cells. The firefly luciferase gene, which is under the control of cAMP response element (CRE), was stably transfected into HEK293 cells. The stable cells were plated on a 96-well plate. On the second day, the cells were induced with different concentrations of foskolin (FSK, adenylyl cyclase activator) for about 16 hours. The firefly luciferase activity was then measured as described in the procedure (without measuring *Renilla* luciferase activity).

II. miTarget™ miRNA Target Sequence 3' UTR Expression Clones

The GeneCopoeia Luc-Pair miR Luciferase Assay Kit was developed and optimized with GeneCopoeia miRNA Target Sequence 3' UTR Expression Clones. The miRNA Target clones offer genome-wide miRNA target sequences with a choice of two mammalian expression vector systems.

The pEZX-MT01 vector shown below, uses the firefly luciferase reporter gene under the control of an SV40 promoter and *Renilla* luciferase as the tracking gene under the control of a CMV promoter. Firefly luciferase expression is regulated by binding of the targeting miRNA to the 3' UTR target sequence and luciferase activity is quantified with a colorimetric assay.

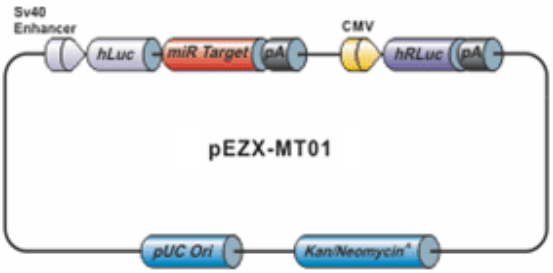


Figure 7. GeneCopoeia miTarget vectors with duo luciferase reporters.

Key advantages of miTarget™ 3' UTR Target Sequence Expression Clones

- The 3' UTR sequences are inserted downstream of coding sequences
- Firefly luciferase and *Renilla* luciferase included on miTarget 3' UTR target clones

III. Contents and Storage

Cat. Nos. LPFR-M010, LPFR-M030 and LPFR-M100

Contents	**Quantity** 100 reactions 300 reactions 1000 reactions	**Shipping temperature**	**Storage temperature**
Luc-Pair miR Luciferase Assay Solution I Lysis and firefly luciferase buffer	10 ml 30 ml 100 ml	Ice pack	–20°C Stable for at least 6 months
Luc-Pair miR Luciferase Substrate I Firefly luciferase substrate	50 µl 150 µl 500 µl	Ice pack	–20°C Stable for at least 6 months
Luc-Pair miR Luciferase Assay Solution II *Renilla* luciferase buffer	10 ml 30 ml 100 ml	Ambient	**Room temperature** Stable for at least 6 months
Luc-Pair miR Luciferase Substrate II *Renilla* luciferase substrate	50 µl 150 µl 500 µl	Ice pack	–20°C Stable for at least 6 months

IV. Preparation

Note 1. Solution I is stable at –20°C for 6 months. Freezing and thawing the reagent can reduce activity of the firefly luciferase. Working Solution I (Solution I combined with Substrate I) is stable at room temperature for several hours. Prepare only the amount of reagent required. For best results, prepare enough reagent immediately before use. For long time storage, store Working Solution I at –70°C.

Note 2. Prepare Working Solution II immediately before use. Store Working Solution II at –70°C.

Note 3. Light intensity is a measure of the rate of catalysis by the luciferases and is therefore temperature sensitive. The temperature optimum for the activity of both luciferases is approximately room temperature (20–25°C), so it is important that the reagents be equilibrated to room temperature before beginning measurements.

A. Microplates

1. Warm up Solution I and Substrate I to room temperature.

2. Calculate the amount of Solution I and Substrate I needed to perform the desired experiments. Dilute Substrate I 1:200 into an appropriate volume of Solution I.
Example: If 6 ml of Solution I is needed, dilute 30 µl of Substrate I into 6 ml of Solution I.

3. Dilute Substrate II 1:200 into an appropriate volume of Solution II.

B. Individual samples

1. Warm up Solution I and Substrate I to room temperature.

2. Prepare 5X Working Solution I: Calculate the amount of Solution I and Substrate I needed to perform the desired experiments. Dilute Substrate I 1:40 into an appropriate volume of Solution I.

Example: If 1 ml of 5X Working Solution I is needed, dilute 25 µl of Substrate I into 1 ml of Solution I.

3. Prepare Working Solution II: Dilute Substrate II 1:200 into an appropriate volume of Solution II.

V. Procedure

A. Microplates

1. **Remove** the desired number of multi-well plates containing mammalian cells from the incubator. Aspirate the cell growth medium.

2. **Measure** firefly luciferase activity.

   Prepare **Working Solution I** by adding 50 µl of Substrate I into 10 ml of Solution I and mix well. For 96-well plates, typically 100 µl of Working Solution I is added to each well. Gently tap the plates several times to efficiently lysis the cells. Do not votex. Wait 10 minutes, measure the firefly luminescence.

   Alternatively, the luciferase assay reagent can be added to cells with the culture medium at a ratio of 2:1, incubated for 10 minutes and assayed for luciferase activity.

3. **Measure** *Renilla* luciferase activity.

   Prepare **Working Solution II** by adding 50 µl of Substrate II into 10 ml of Solution II and mix well. For 96-well plates, typically 100 µl of Working Solution II is added to each well already containing 100 µl of Working Solution I.

   Gently tap the plates several times to mix. Wait 10 minutes, measure the *Renilla* luminescence.

4. **Calculate** the ratio of luminescence from the firefly luciferase to the *Renilla* luciferase.

IMPORTANT NOTE: Because the luminescent signals are affected by assay conditions, raw results should be compared only between samples measured at the same time and using the same medium/serum combination. Incorporation of consistent control wells on each plate provides the ability to calculate a normalized firefly luminescence/*Renilla* luminescence ratio for each sample well. These normalized ratios will remain essentially constant (±10%) for samples in a plate measured during the 1-hour measurement window.

B. Individual samples

1. Set up the luminometer as below before beginning the experiment:

Volume injection #1:	20 (µl)
Volume injection #2:	100 (µl)
Measuring background	Yes
Background measuring time	0.5 s
Automatic BKG subtract	Yes
Max BKG [RLU/s]	50
Delay INJ. 1/MEAS. 1	2 s
Delay INJ. 2/MEAS. 2	2 s
Measuring time 1	2 s
Measuring time 2	2 s
Number of replicates	1 s
Normalized response	M1/M2

2. Remove the mammalian cell culture from the incubator. Aspirate the cell growth medium.

3. Add Solution I (without Substrate I) into the cultured cells as indicated below:

Culture vessel	Surface area (cm^2)	Solution I added
96-well plate (per well)	0.3	80 (µl)
24-well plate (per well)	1.9	320 (µl)
12-well plate (per well)	4	640 (µl)
6-well plate (per well)	9.3	1.2 (ml)
35-mm dish	7.5	1.2 (ml)
6-cm dish	21	3 (ml)
10-cm dish	49	4 (ml)

IMPORTANT NOTE: Tap the plates several times to efficiently lysis the cells. Inefficiently lysis of the cells will cause a low reading in the assays.

4. Wait for 3 minutes. Transfer 80 µl of the cell lysate into a tube.

5. Measuring firefly luciferase and *Renilla* luciferase activities.

   Injection #1 is 5X Working Solution I
   Injection #2 is Working Solution II

VI. References

1. deWet, JR et al. (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli. Proc. Natl. Acad. Sci.USA 82: 7870–7873.

2. Wood, KV (1991) In: Bioluminescence and Chemiluminescence: Current Status, Stanley, P., and Kricka, L., eds., John Wiley and Sons, Chichester, NY, 543.

3. Alam, J et al. (1990) Reporter genes: Application to the study of mammalian gene transcription. Anal. Biochem. 188: 245–54.

4. Matthews, JC et al. (1977) Purification and properties of *Renilla* reniformis luciferase. Biochemistry 16: 85–91.

VII. Limited Use License and Warranty

Limited Use License
Following terms and conditions apply to use of the Luc-Pair™ miR Luciferase Assay Kit (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products or deliver information obtained in service without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty
GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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